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APPELLANTS' BRIEF Address to: Mail Stop Appeal Brief-Patents Commissioner for Patents P.O. Box 1450 Alexandria, VA 22313-1450	Application Number	10/001,688
	Confirmation Number	3172
	Attorney Docket No.	10010819-1
	Filing Date	October 25, 2001
	First Named Inventor	Theodore Sana
	Examiner	Joyce Tung
	Group Art	1637
	Title: *Compositions and Methods for Optimized Hybridization Using Modifier Solutions*	

Sir:

This Brief is filed in support of Applicants' appeal from the Examiner's Rejection dated February 24, 2005. No claims have been allowed, and Claims 6-8 and 15-24 are pending. Claims 6-8 and 15-24 are appealed. A Notice of Appeal was filed on April 22, 2005. A petition for a one-month extension of time is filed herewith, making this Appeal Brief timely filed.

The Board of Appeals and Interferences has jurisdiction over this appeal pursuant to 35 U.S.C. §134.

The Commissioner is hereby authorized to charge deposit account number 50-1078, reference no. 10010819-1 to cover any fee required under 37 C.F.R. §1.17(c) for filing Applicants' brief. In the unlikely event that the fee transmittal or other papers are separated from this document and/or other fees or relief are required, Applicants petition for such relief, including extensions of time, and authorize the Commissioner to charge any fees under 37 C.F.R. §§ 1.16, 1.17 and 1.21 which may be required by this paper, or to credit any overpayment, to deposit account number 50-1078, reference no. 10010819-1.

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TABLE OF CONTENTS

REAL PARTY IN INTEREST

The inventors named on this patent application assigned their entire rights to the invention to Agilent Technologies, Inc.

RELATED APPEALS AND INTERFERENCES

There are currently no other appeals or interferences known to Appellants, the undersigned Appellants' representative, or the assignee to whom the inventors assigned their rights in the instant case, which would directly affect or be directly affected by, or have a bearing on the Board's decision in the instant appeal.

STATUS OF CLAIMS

The present application was filed on October 25, 2001 with claims 1-17. In response to a restriction requirement, claims 6-8 and 15-17 were elected for further prosecution. Claims 1-5 and 9-14 were later canceled. Claims 18-24 were added. During the course of prosecution, Claims 6-8 and 15-19 were amended. Claim 19 was allowed but then later rejected for being indefinite. Accordingly, Claims 6-8 and 15-24 are pending in the present application, all of which are appealed herein.

STATUS OF AMENDMENTS

No amendments to the Claims were filed subsequent to issuance of the Final Rejection.

SUMMARY OF CLAIMED SUBJECT MATTER

The claimed invention is drawn to a method for allowing a probe and a target to specifically hybridize at a lower temperature than their standard hybridization temperature. In the subject methods, an oligonucleotide probe that is covalently linked to the surface of a microarray is heated along with a target in the presence of a chemical component of the formula: $R(NH_2)C=O$, where R is an amino or a methyl group (e.g., acetamide), and allowed to hybridize. The subject method finds use in a variety of different applications, including the detection of nucleic acids.

Below is a description of each appealed claim and where support for each can

be found in the specification (listed in parentheses).

Independent Claim 6 claims a method that allows a probe and target to specifically hybridize at a temperature lower than their standard hybridization temperature, which includes: (a) heating the probe and target in the presence of a chemical component of the formula: $R(NH_2)C=O$, where R is an amino or a methyl group (specification at page 2, lines 17-23); and (b) allowing the probe and target to hybridize (specification at page 2, lines 25-28), wherein the probe is an oligonucleotide probe covalently linked to the surface of a microarray (specification at page 6, lines 10-14 and Example 1).

Claim 7 claims a method according to Claim 6, wherein the probe and target are heated to a temperature that is lower than their standard hybridization temperature. (See the specification at page 13, lines 5-15 and page 20, lines 18-20.)

Claim 8 claims a method according to Claim 6, which specifies that the chemical component is to be added to a solution prior to heating the probe and target (i.e., step (a)). (See the specification at page 2, line 26.)

Independent Claim 15 claims a method that allows a probe on a micro array surface to specifically hybridize to a target at a temperature lower than their standard hybridization temperature, which includes: (a) heating the probe and target in the presence of a chemical component of the formula: $R(NH_2)C=O$, where R is an amino or a methyl group (specification at page 10, lines 4-14); and (b) allowing the probe and target to hybridize (specification at page 10, lines 15-17), wherein said probe is an oligonucleotide probe covalently linked to the surface of a microarray. (See Example 2).

Claim 16 claims a method according to Claim 15, wherein the probe and the target are heated to a temperature that is lower than their standard hybridization temperature. (See the specification at page 9, lines 7-15.)

Claim 17 claims a method according to Claim 15, which specifies that the chemical component is to be added to a solution prior to heating the probe and target (i.e., step (a)). (See the specification at page 14, line 30 to page 15, line 2.)

Claim 18 claims a method according to Claim 6, wherein the chemical component is urea. (See the specification at page 20, line 9.)

Independent Claim 19 claims a method that allows a probe and target to hybridize at a temperature lower than their standard hybridization temperature, which includes: (a) heating the probe and target in the presence of acetamide (specification at page 10, lines 10-11); and (b) allowing the probe and target to hybridize (specification at page 19, lines 23-25), wherein the probe is an oligonucleotide probe attached to the surface of a glass substrate (specification at page 15, line 11).

Independent Claim 20 claims a method for detecting nucleic acids using a microarray that includes: (a) contacting a sample comprising labeled nucleic acids with an addressable microarray of oligonucleotide probes covalently linked to a surface of a glass substrate in a hybridization buffer comprising urea (specification at page 5, line 4); and detecting any labeled nucleic acids that specifically hybridize to the oligonucleotide probes. (See the specification at page 7, line 18.)

Claim 21 claims a method according to Claim 20, wherein the contacting is done at a hybridization temperature of about 50°C. (See the specification at page 11, line 12.)

Claim 22 claims a method according to Claim 20, wherein urea is present in the hybridization buffer at a concentration of about 5M. (See the specification at page 13, line 5.)

Claim 23 claims a method according to Claim 20, wherein urea is present in

the hybridization buffer at a concentration of about 4M. (See the specification at page 13, line 17.)

Claim 24 claims a method of Claim 20, wherein the oligonucleotides are 60-mer oligonucleotides. (See the specification at page 13, line 20.)

GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

I. Claims 6-8 and 15-23 are rejected under 35 USC §112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicants regard as the invention. The Examiner asserts that the term "oligonucleotide" is unclear.

II. Claims 6-8, 15-18 and 20-24 are rejected under 35 USC § 103(a) as being unpatentable over Brenner (US Patent No. 5,604,097).

ARGUMENT

I. Claims 6-8 and 15-23 are rejected under 35 USC §112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicants regard as the invention.

The Appellants wish to group the Claims as follows: Claims 6-8 and 15-18 as a first group; Claim 19 as a second group; and Claims 20-23 as a third group.

In this rejection, the Examiner asserts that Claims 6-8 and 15-23 are indefinite for their recitation of the term "oligonucleotide probe." In making this rejection, the Examiner asserts that it is unclear what the size of the oligonucleotide probe is and because there is no definition of "oligonucleotide probe" in the specification clarification is necessary.

In response, the Applicants respectfully submit that the meaning of the terms "oligonucleotide" and "probe" are clear to one of skill in the art. According to the MPEP

at § 2171, all that is required to comply with the requirements of 35 U.S.C. § 112, second paragraph, is that the Applicant show that the scope of the claim is clear to a person possessing the ordinary level of skill in the pertinent art.

Group I: Claims 6-8 and 15-18

The claimed invention of this group is directed to methods for allowing a probe and target to hybridize at a temperature lower than their standard hybridization temperature, wherein the probe is an oligonucleotide, covalently linked to the surface of a microarray, and it is heated with a target in the presence of a chemical component of the formula: $R(NH_2)C=O$.

The term "oligonucleotide" is found in over 129,000 abstracts in NCBI's PubMed database, in the specification of over 33,000 issued patents, and in the claims of over 4,500 issued patents. The term "oligonucleotide" is defined in several dictionaries, is a term that is widely used by molecular biologists, and is explicitly defined in the instant specification. (See page 4, lines 30-33). The specification clearly teaches that an oligonucleotide refers to a nucleotide multimer of about 10 to about 100 nucleotides in length.

The term "probe" with reference to an oligonucleotide is found in the specification of about 7,000 issued patents and in the claims of over 500 issued patents. The specification clearly teaches a probe refers to a nucleic acid. (See the specification at page 5, lines 20-23.) Additionally, with reference to FIG. 9, the specification sets forth oligonucleotide probes of increasing length: 20, 25, 30, 35, 40, 45, 50, 55 and 60-mers. (See the specification page 14, lines 3-4.)

Given this description in the specification, the subject matter embraced by the term "oligonucleotide probe" is clear and is not of a scope that is beyond that which is commonly understood in the art and defined in the specification. Specifically, an "oligonucleotide probe" means an "oligonucleotide" that is between 10 and 100 nucleotides in length that functions as a "probe".

The MPEP at § 2173.04 teaches that the breadth of a claim is not to be equated with indefiniteness. If the scope of the subject matter embraced by the claims is clear, and if applicants have not otherwise indicated that they intend the invention to be of a scope different from that defined in the claims, then the claims comply with 35 U.S.C. §112, second paragraph.

In light of the prior art, and in view of the specification, the term "oligonucleotide probe" is not a term that is subject to being misunderstood by one of skill in the art, as one of skill in the art would instantly know what an oligonucleotide probe is and would, therefore, recognize what is being claimed without ambiguity. Hence, the rejected claims comply with 35 U.S.C. §112, second paragraph, and the Applicant respectfully requests the withdrawal of the rejection.

Group II: Claim 19

The claimed invention of this group is directed to a method for allowing a probe and target to hybridize at a temperature lower than their standard hybridization temperature, wherein the probe is an oligonucleotide and it is heated with a target in the presence of acetamide.

In addition to the arguments detailed above for the Claims of Group I, the Applicant would like to further point out, with respect to Claim 19, that in the October 23, 2003 and April 5, 2004 Office Actions, Claim 19 was allowed. However, subsequent to this allowance, and without being amended by the Applicant, the Office has now rejected Claim 19 as being indefinite with respect to use of the term "Oligonucleotide probe." The Applicant, therefore, respectfully requests this rejection be withdrawn.

Group III: Claims 20-23

The claimed invention of this group is directed to a method for detecting nucleic acids using a microarray that includes oligonucleotide probes and a hybridization buffer that comprises urea.

In addition to the arguments detailed above for the Claims of Group I, the Applicant would like to further point out, with respect to Claims 20-23, that the use of microarrays of oligonucleotide probes is a common research tool that is widely practiced in the scientific community. According to the MPEP § 2163, an Applicant need not set forth in precise terms what is conventional in the art or known to one of ordinary skill. Accordingly, in light of the arguments set forth with respect to the Claims of Group I and in conjunction with the above, the Applicant has set forth without ambiguity what is meant by the term "oligonucleotide probe," and assuming arguendo that this is not the case, which it is, in light of MPEP § 2163 the Applicant need not set forth precisely what is meant by the term "oligonucleotide probe" as the term is conventional and well known in the art.

II. Claims 6-8, 15-18 and 20-24 are rejected under 35 USC § 103(a) as being unpatentable over Brenner (US Patent No. 5,604,097) in view of Oliva et al. (BioTechniques, 2001, Vol. 31(1) pgs 74-76 and 78-81).

As stated in MPEP § 2143:

> To establish a *prima facie* case of obviousness, three basic criteria must be met. First, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings. Second, there must be a reasonable expectation of success. Finally, the prior art reference (or references when combined) must teach or suggest all the claim limitations.

The Examiner asserts that the teachings of Brenner combined with the teachings of Oliva et al. render the claimed invention obvious. The Examiner asserts that Brenner teaches a method of tracking, identifying and sorting classes of molecules by use of oligonucleotide tags that are covalently immobilized on a solid support (citing Brenner's specification column 12, lines 40-47, column 13, lines 7-9, and column 35, lines 1-18). The Examiner further asserts that Brenner sets forth oligonucleotide tags that are from 12 to 60 nucleotides in length.

The Examiner concedes that Brenner does not teach a hybridization reaction performed in the presence of urea at a concentration of 4M at about 50° C. Hence, the Examiner turns to Oliva et al. to fill this deficiency. The Examiner asserts that Oliva teaches hybridization with a urea buffer of 4M at 50° C. The Examiner further asserts that it would have been obvious to modify the specific hybridization methods of Brenner by applying the Urea buffer of Oliva et al. The Applicant respectfully traverses.

The Appellants wish to group the Claims as follows: Claims 6-8 and 15-18 as a first group; Claim 19 as a second group; and Claims 20-23 as a third group. Below are the contentions of the Appellant with respect to this ground of rejection, with a separate subheading for each group of claims.

Group I: Claims 6-8 and 15-18

The claimed invention of this group is directed to methods for allowing a probe and target to hybridize at a temperature lower than their standard hybridization temperature, wherein the probe is an oligonucleotide probe, covalently linked to the surface of a microarray, and it is heated with a target in the presence of a chemical component of the formula: $R(NH_2)C=O$. The Applicant respectfully requests reconsideration and withdrawal of the rejection of the claims in this group under 35 USC § 103(a) for the following reasons.

According to the MPEP § 1504.03 when a modification to a basic reference involves a change in configuration, both the basic and secondary references must be from analogous arts. The reason is that under 35 U.S.C. 103(a), a designer of ordinary skill would not be charged with knowledge of prior art that is not analogous to the claimed design.

Brenner is not capable of being combined with Oliva in the manner suggested by the Examiner because Brenner and Oliva constitute non-analogous art. Brenner discloses a method in which a compound is labeled with an oligonucleotide tag, the labeled compound is contacted with a sample, and labeled compounds that are bound to sample are identified by means of the oligonucleotide tag binding to its complement that is attached to a solid support. Oliva is directed to co-localization studies involving Green Fluorescent Protein and GAD67 mRNA in situ hybridization. In practicing the methods disclosed therein, a tissue sample is first obtained and prepared, then it is embedded in paraffin, then in situ hybridization and co-localization assays are performed and the tissue samples are photographed. In situ hybridization assays are completely different from hybridization assays carried out using an oligonucleotide probe attached to the surface of a solid support, such as a micro array. Not only are the protocols dissimilar, the conditions under which the two hybridization reactions take place are significantly different. The hybridization reactions set forth in Oliva are performed on free-floating sections of tissue samples, and therefore, do not involve oligonucleotide probes that are bound to a solid support. Accordingly, one of skill in

the art facing a problem in performing a micro array based assay would not readily look to the In Situ arts for guidance.

According to the MPEP § 2144.05 a *prima facie* case of obviousness may be rebutted by showing that the art, in any material respect, teaches away from the claimed invention.

Furthermore, assuming arguendo, that one of skill in the art would look to the teachings of both Brenner and Oliva, a *prima facie* case for obviousness can still not be maintained because Brenner actually teaches away from the claimed invention, and therefore, cannot be properly combined with Oliva to render the claimed invention obvious.

Specifically, Brenner's specification at col. 2, lines 22-28 warns that the use of reagents that alter base-specific stability of nucleic acid duplexes in a hybridization assay would be undesirable because their effects are limited and can be incompatible with further manipulations. Urea is an example of a reagent that "alters base-specific stability of nucleic acid duplexes", and as such would be excluded from use in the methods of Brenner.

Additionally, Brenner's specification at col. 2, lines 44-50 presents his invention as a system which "minimized the occurrence of false positives and false negative signals without the need to employ special reagents for altering natural base pairing". Again, urea is a reagent that alters natural base pairing, and as such should be excluded from use in the methods of Brenner. Accordingly, Brenner explicitly warns against the use of reagents that alter base-specific stability of nucleic acid duplexes

In view of Brenner's extensive teachings against the use of reagents that alter base pairing in a hybridization buffer, one of skill in the art would not combine the urea of Oliva, a reagent that alters base pairing, into Brenner's methods.

According to the MPEP § 2143 there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings.

Further still, assuming arguendo that Brenner could be properly combined with Oliva, which it cannot be, a *prima facie* case of obviousness cannot be established because there is no motivation to combine the two references in the manner suggested by the Examiner. Simply because two references can be combined is not suggestive that they should be combined. In this instance, neither reference alone nor in combination suggests the presently claimed invention nor sets forth any reason as to why one of ordinary skill in the art would be motivated to combine them.

The Applicant's presently claimed invention is directed to the use of urea in a hybridization assay carried out on an oligonucleotide array. The problem that the Applicant was trying to solve is related to lowering the hybridization temperature required for a nucleic acid probe of increased length to hybridize to a target molecule. As probe length increases, the temperature required for hybridization also increases. This increased temperature may be detrimental to the micro array surface. (See the specification at page 9, lines 15-21.) Neither of the cited references recognizes the relationship between increased probe length and increased hybridization temperature, nor do they recognize that this increased temperature may lead to a loss of probe sensitivity and may be detrimental to the array surface. Accordingly, neither reference alone nor in combination sets froth the use of urea as a means of lowering hybridization temperatures in array based hybridization assays. Without recognizing the need and without asserting the Applicant's solution to that need, there can be no suggestion of, nor motivation to combine the two references to derive, the Applicant's claimed invention.

In the instant rejection, the Office has used impermissible hindsight gained from the Applicant's specification to combine the unrelated references and has argued that because the combination of the two references could yield the favorable result of

reduced hybridization temperature (which is the subject matter claimed in the Applicant's invention), the Applicant's invention must necessarily be rendered obvious. However, Brenner discloses the use of oligonucleotide tags for the labeling and sorting of target molecules. Except for saying that the hybridization conditions should be stringent, Brenner does not discuss any factors affecting the hybridization conditions used. Oliva is directed to co-localization studies involving Green Fluorescent Protein and GAD67 mRNA in situ hybridization. Neither reference is directed to solving the problem the Applicant set out to solve, neither reference suggests the Applicant's claimed invention, and neither reference sets forth why one of ordinary skill in the art would look to the other reference for a solution to that problem.

A *prima facie* case of obviousness may be rebutted by a showing of unexpected results. According to the MPEP § 2141 objective evidence or secondary considerations such as unexpected results, commercial success, long-felt need, failure of others, copying by others, licensing, and skepticism of experts are relevant to the issue of obviousness and must be considered in every case in which they are present.

Assuming arguendo, that a *prima facie* case of obviousness could be established, which it cannot, the Applicants hereby rebut that presumption. The Applicants submit that the claimed method provides unexpected results.

The claimed urea-based methods provide enhanced results which could not have been predicted from the teachings of the cited art, in comparison to equivalent methods using other denaturants. As evidence of these advantages, Applicants have attached herewith a post-filing publication demonstrating the unexpected benefits of the presently claimed methods and compositions. The date appearing on this publication is "October 2002", well after the filing date of the instant application.

This publication is a brochure from MWG Biotech AG describing hybridizing a microarray of oligonucleotide probes with target nucleic acids from rat liver and kidney, in a variety of different denaturants, including salt (e.g., SSC and SSPE), two different concentrations of formamide, and urea. The results from this experiment are shown on

page 2 of the publication in the graph entitled Fig. 1. Quoting from the publication: "The slide-to-slide correlation of ratios were clearly better in urea-buffer (see Figure 2)."

These data demonstrate that urea buffers are superior to buffers containing other denaturants (even those buffers containing salt) in oligonucleotide microarray experiments. This superiority could not have been predicted by the teachings of Brenner or Oliva et al. Accordingly, one of skill in the art could not have predicted the success of the presently claimed invention.

Therefore, because the combined teachings of Brenner and Oliva et al. fail to teach or suggest each and every element of the claimed invention, a *prima facie* case of obviousness has not been established. And even if a *prima facie* case of obviousness could be established, which it cannot be, the Applicant has rebutted that presumption by showing unexpected results. The Applicant, therefore, respectfully request withdrawal of this rejection.

Group II: Claim 19

The claim in this group was not rejected under these grounds.

Group III: Claims 20-24

The claimed invention of this group is directed to a method for detecting nucleic acids using a microarray that includes oligonucleotide probes and a hybridization buffer that comprises urea. The Applicant respectfully requests reconsideration and withdrawal of the rejection of the claims in this group under 35 USC § 103(a) for the following reasons.

In addition to the arguments detailed above for the Claims of Group I, the Applicant would like to further point out, with respect to Claims 20-24, that these rejected claims are directed to the use of nucleic acid microarrays containing bound oligonucleotide probes for use in detecting nucleic acid targets. As such, Oliva, which is directed to in situ co-localization experiments involving isolated and free-floating tissue samples is not in the same field as Applicant's and therefore constitutes non-analogous

art that should not be combined with Brenner et al. to render the claimed invention obvious. Additionally, Claims 21-24 set out hybridization temperatures, buffer concentrations, and probe lengths that are not taught or fairly suggested by either reference, alone or in combination. Brenner does not set forth reaction temperatures or the use of urea at all, and Oliva teaches the use of ambient temperatures, a urea concentration of 1 M, and does not set forth the probe lengths used. Accordingly, the Applicant, respectfully request the withdrawal of this rejection.

SUMMARY

As detailed in the Arguments section above, the Applicants submit that:

Claims 6-8 and 15-23 are not indefinite under 35 USC §112, second paragraph, for being indefinite; and

Claims 6-8, 15-18 and 20-24 are not unpatentable under 35 USC §103(a) over Brenner (US Patent No. 5,604,097) in view of Oliva et al. (BioTechniques, 2001, Vol. 31(1) pgs 74-76 and 78-81).

RELIEF REQUESTED

The Applicants respectfully request that all rejections of Claims 6-8 and 15-24 be reversed and that the application be remanded to the Examiner with instructions to issue a Notice of Allowance.

Respectfully submitted,

Date: 7.25.05

By: _____
Bret Field
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CLAIMS APPENDIX

6. A method that allows a probe and target to specifically hybridize at a temperature lower than their standard hybridization temperature, comprising:

(a) heating the probe and target in the presence of a chemical component of the formula:

$R(NH_2)C=O$

where R is an amino or a methyl group; and

(b) allowing the probe and target to hybridize,

wherein said probe is an oligonucleotide probe covalently linked the surface of a microarray.

7. A method as recited in claim 6, wherein said probe and target are heated to a temperature that is lower than their standard hybridization temperature.

8. A method as recited in claim 6, further comprising adding said chemical compound to a solution prior to heating step (a).

15. A method that allows a probe on a micro array surface to specifically hybridize to a target at a temperature lower than their standard hybridization temperature, comprising:

(a) heating the probe and target in the presence of a chemical component of the formula:

$R(NH_2)C=O$

where R is an amino or a methyl group; and

(b) allowing the probe and target to hybridize,

wherein said probe is an oligonucleotide probe covalently linked to the surface of a microarray.

16. A method as recited in claim 15, wherein said probe and target are heated to a temperature that is lower than their standard hybridization temperature.

17. A method as recited in claim 15, further comprising adding said chemical compound to a solution prior to heating step (a).

18. A method as recited in claim 6, wherein said chemical component is urea.

19. A method that allows a probe and target to hybridize at a temperature lower than their standard hybridization temperature, comprising:

(a) heating the probe and target in the presence of acetamide; and

(b) allowing the probe and target to hybridize,

wherein said probe is an oligonucleotide probe attached to the surface of a glass substrate.

20. A method for detecting nucleic acids using a microarray, comprising:

contacting a sample comprising labeled nucleic acids with an addressable microarray of oligonucleotide probes covalently linked to a surface of a glass substrate in a hybridization buffer comprising urea; and

detecting labeled nucleic acids that specifically hybridize to said oligonucleotide probes.

21. The method of claim 20, wherein said contacting is done at a hybridization temperature of about 50°C.

22. The method of claim 20, wherein urea is present in said hybridization buffer at a concentration of about 5M.

23. The method of claim 20, wherein urea is present in said hybridization buffer at a concentration of about 4M.

24. The method of claim 20, wherein said oligonucleotides are 60-mer oligonucleotides.

EVIDENCE APPENDIX

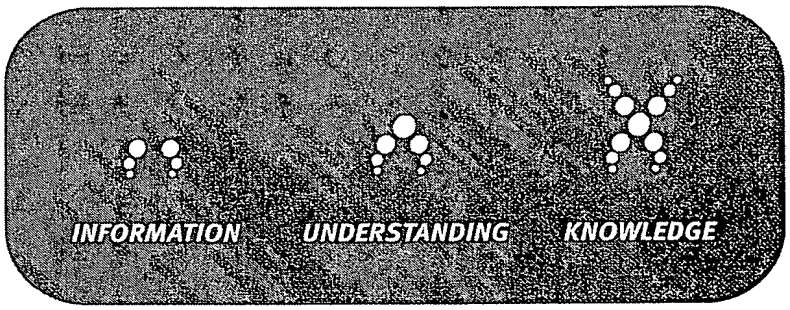
A post-filing publication demonstrating the unexpected benefits of the presently claimed methods and compositions is herein attached as Exhibit A. This publication is a brochure from MWG Biotech AG describing hybridizing a microarray of oligonucleotide probes with target nucleic acids from rat liver and kidney, in a variety of different denaturants, including salt, two different concentrations of formamide, and urea. As can be seen, hybridization results were better in the urea-buffer. See Figure 1.

RELATED PROCEEDINGS APPENDIX

As stated in the *Related Appeals and Interferences* section above, there are no other appeals or interferences known to Appellants, the undersigned Appellants' representative, or the assignee to whom the inventors assigned their rights in the instant case, which would directly affect or be directly affected by, or have a bearing on the Board's decision in the instant appeal. As such this section is left blank.

Dajana Preuß, Mirjam Gördes, Gabie Seidl, Jutta Siebert, Michael Bergmann, Dr. Andrea Huber, and Dr. Susanne Schröder
MWG Biotech AG, Genomic Diagnosis, Anzinger Str. 7a, 85560 Ebersberg, Germany

THE DEVELOPMENT OF A HYBRIDIZATION BUFFER FOR LARGE-VOLUME HYBRIDIZATIONS



MWGd
GENOMIC DIAGNOSIS

The complete automation of the hybridization process including sample preparation is an important long-term goal for allowing high throughput in terms of maximum reproducibility and quality assurance. This study focuses on the hybridization process itself. In order to allow hybridization in large volumes (70-350µl) – as it is necessary for most automated hybridization chambers – we had to develop and optimize such a system. Moreover, hybridization in a larger volume is supposed to generate more reproducible results. Consequently we found that primarily the development of a hybridization buffer allowing stringent and sensitive, reproducible hybridizations was necessary.

As a test system, the MWG Rat5K Array was used and hybridized competitively using rat tissue (liver against kidney). This array contains 5535 Oligos (50 mers) representing ORFs, 100 replicated spots and 125 Arabidopsis control oligos. Hybridization samples were generated by direct labeling and amplification. The hybridizations were carried out using MWG Gene Frames specially designed for MWG arrays in order to simulate the large volume approach. Using these frames a MWG Rat 5K Array is hybridized in 240µl probe volume.

Denaturing agents

A hybridization buffer can be a pure salt based buffer. Different salts are possible, the most common are SSC and SSPE. Their concentration depends on the favored stringency. Formamide is also frequently added because of its strong denaturing features, although it may cause problems for large volume hybridizations. Alternatives to formamide would be urea, which is much less toxic, and guanidine-hydrochloride, a chaotrophic salt, often used in DNA-kits. This study focused on the comparison of formamide and urea. A 50% formamide-buffer hybridized under a coverslip was used as a reference. Figure 1 shows a profile display comparing the ratios derived from experiments using different denaturing agents in the hybridization buffer. The results show that ratios were compressed in pure salt-based buffer, whereas lowering the formamide concentration to 30% yielded comparable results to the reference. In urea containing buffer ratios were even spread under the same hybridization conditions.

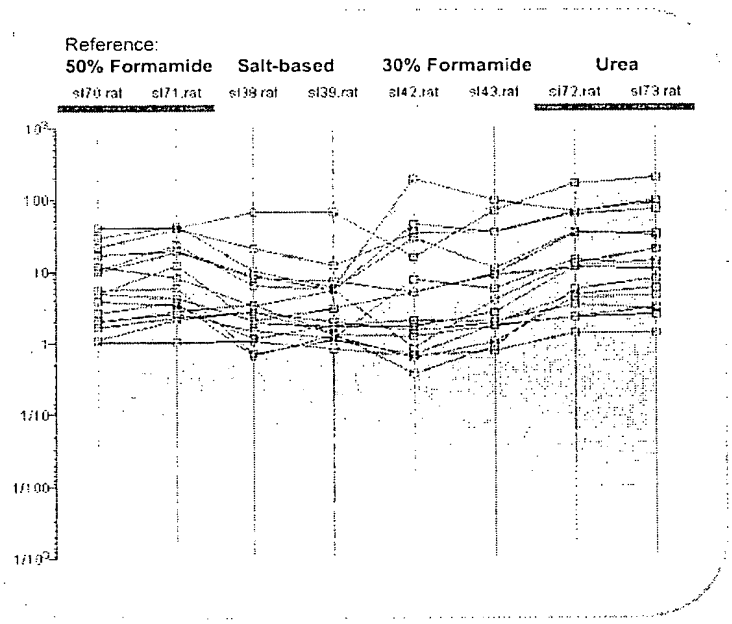


Figure 1. Denaturing agents: Profile Display. This figure shows a profile display comparing the ratios derived from experiments using different denaturing agents in the hybridization buffer. The highlighted genes represent a selection which is expressed in liver. The first two slides were hybridized under coverslips using a 50% formamide buffer (reference approach).

The slide-to-slide correlation of ratios were clearly better in urea-buffer (see Figure 2). Here, 98.9% of the values varied within a twofold range. These were only 89.7% in 30% formamide-buffer. However, signal intensities were significantly lower with both buffers than with the reference approach.

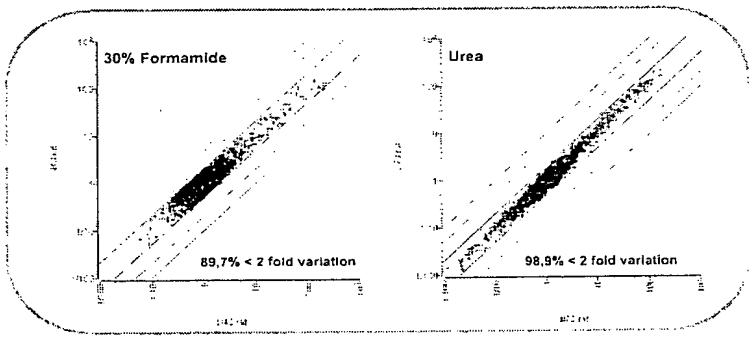


Figure 2. Denaturing agents: Slide-to-slide correlation. Log/log blots expressing the slide-to-slide correlation of ratios in buffers containing 30% formamide and urea, respectively. Ratios varying within a twofold range are marked red.

Signal enhancement

Thus, in order to combine the specificity of buffers containing denaturing agents with sensitivity, the next step was to focus on signal enhancement strategies. The addition of dextran sulfate for instance, results in a virtual volume reduction effect. In aqueous solutions it is strongly hydrated. Therefore macromolecules have no access to the hydrating water which causes an apparent increase in probe concentration and consequently higher hybridization rates. Another possibility would be the decrease of the concentration of denaturing agents. As this results in reduced stringency, the concentration has to be adapted carefully. In order to increase diffusion of the sample over the array, the assay can be agitated during the incubation time. The easiest possibility is the incubation in a shaking waterbath. The effect of the addition of 5% dextran sulfate is shown in Figure 3 in direct comparison to an array hybridized without it.

The addition obviously resulted in a very strong signal increase because of the volume reduction effect. An increased background and gradient formation were also frequently observed. Moreover, the specificity was significantly decreased. Therefore this approach was rejected.

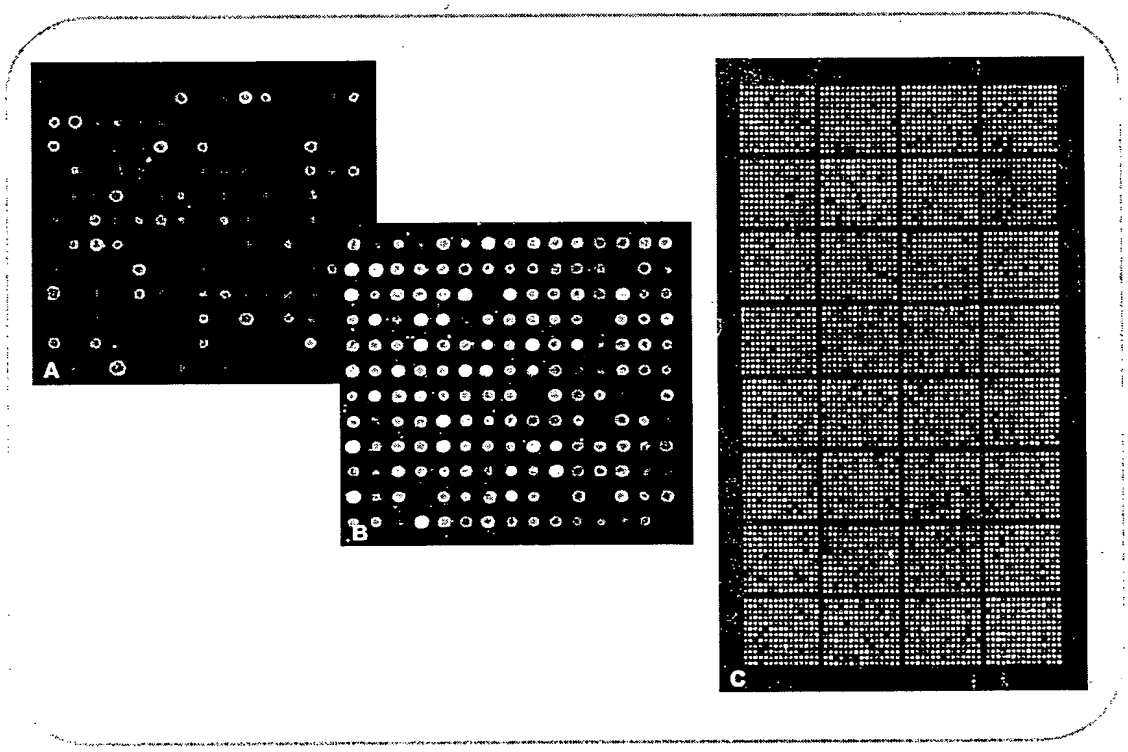


Figure 3. Signal enhancement: Dextran sulfate. This figure shows the effect of the addition of 5% dextran sulfate (B, C) in direct comparison to an array hybridized without it (A).

A decreasing concentration of formamide from 30% to 10% led to significantly increased signals (three-/fourfold) as shown in Figure 4.

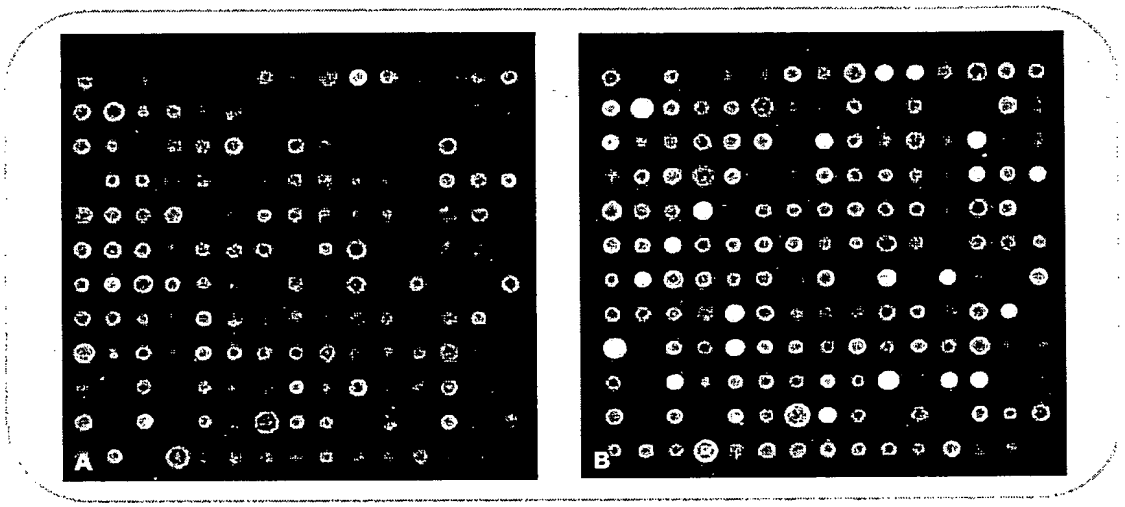


Figure 4. Signal enhancement: Formamide concentration. This figure shows the effect of a decreasing concentration of formamide from 30% (A) to 10% (B).

However, as shown in the profile display of the ratios derived from these two experiments (see Figure 5), the specificity was also decreased. Due to the significant loss of specificity, this step was also not successful.

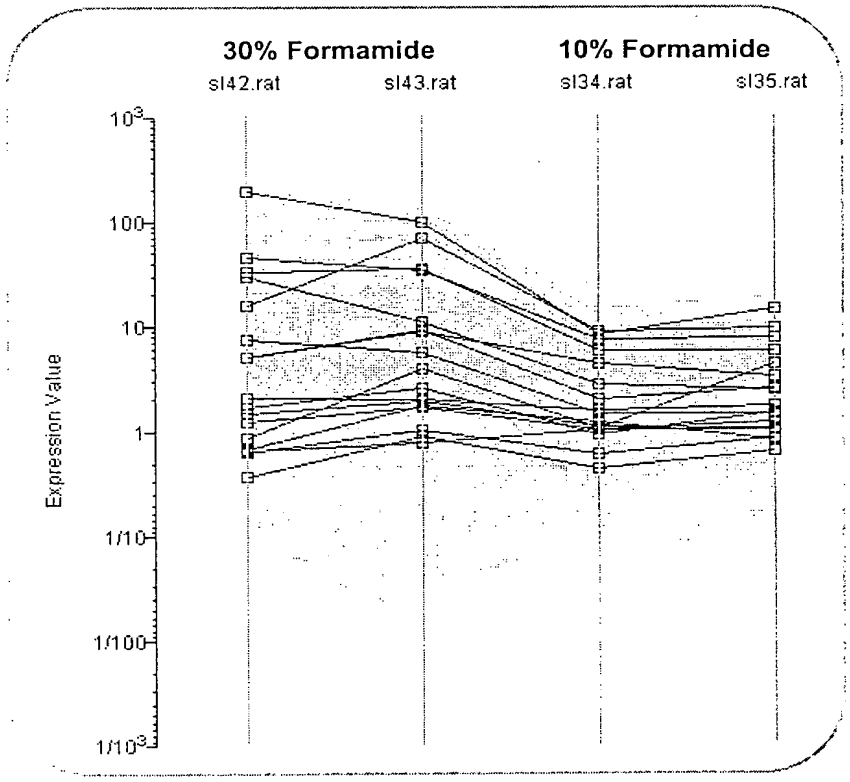


Figure 5. Signal enhancement: Formamide concentration. This figure shows a profile display of the ratios derived from the two experiments reducing the formamide concentration. The highlighted genes represent a selection which is expressed in liver.

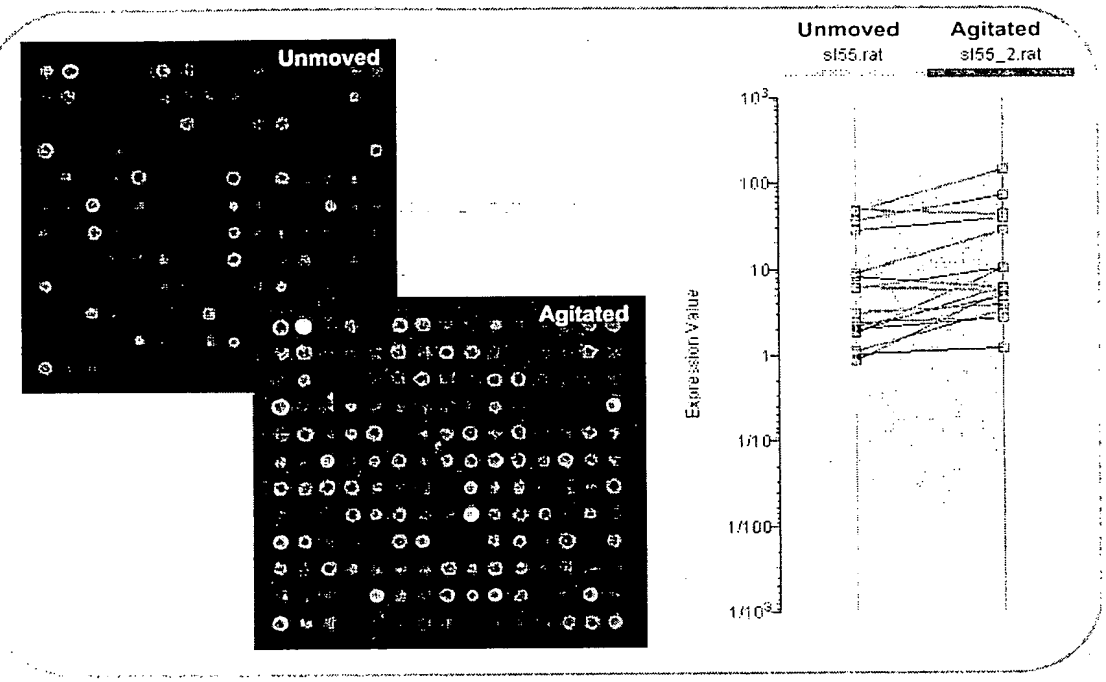


Figure 6. Signal enhancement: Agitation. This figure shows the influence of agitated hybridization by incubation in a shaking waterbath on signal intensities (left) and ratios (right). The highlighted genes represent a selection which is expressed in liver.

Meanwhile, agitation by incubation in a shaking waterbath also led to signal enhancement as shown in Figure 6 in direct comparison of the two slides. Thereby, the specificity was also slightly increased because of the enhanced diffusion (profile display of the ratios in Figure 6). In total, the increase of signal intensity was less strong than that achieved by lowering the formamide concentration. But as the specificity was even positively affected, this approach was favored.

Stringent conditions

An important factor during the improvement of the hybridization process are the hybridization conditions and their respective stringency. First the hybridization temperature was varied in a range of 42 up to 55°C. Increasing temperature generally leads to increased stringency but background problems on the slides also tend to occur the higher the temperature is chosen. The increase of wash temperature has the same impact. Second, the salt concentrations in hybridization and wash buffers can be lowered, but the influence on stringency is not that significant than those of the other adjustments. Another point as already mentioned, is agitation during the hybridization process. This has a positive influence on stringency and will moreover enhance signal intensities.

Strategy and Solution

The strategy for developing a specific and sensitive hybridization buffer can thus be

- to use a pure salt-based buffer, or
- to add denaturing agents.

In the first case a careful adjustment of hybridization conditions (hybridization and wash temperature, salt concentration in hybridization and wash buffer) would be necessary to achieve an optimal balance between specificity and sensitivity. If denaturing agents are used, their concentration has to be carefully adjusted. The results of our study clearly show that the second option was most effective. Thus the solution in our case was a hybridization buffer containing a denaturing agent, precisely an urea-based buffer, because of the lower toxicity and better slide-to-slide reproducibility compared to formamide. In addition agitation during incubation appeared to be recommended.

Conclusion and perspective

In conclusion it can be stated that a hybridization system was created in this study which allows a good imitation of automated hybridization. Moreover, future automation of the hybridization process

- will reduce variations due to manual handling, and thus further increase reproducibility
- and will probably also increase signal intensities and significantly reduce hybridization times due to the agitation of the hybridization solution.

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